Award Number:

W81XWH-13-2-0016

TITLE:

Development of in vivo Biomarkers for Progressive Tau Pathology after Traumatic Brain Injury

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REPORT DATE:

February 2014

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
February 2014	Annual	15 JAN 2013- 14 Jan 2014

4. TITLE AND SUBTITLE

Development of in vivo Biomarkers for Progressive Tau Pathology after Traumatic Brain Injury

5a. CONTRACT NUMBER
W81XWH-13-2-0016

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Washington University
660 S Euclid Ave
St Louis, MO 63110

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Athletes in contact sports who have sustained multiple concussive traumatic brain injuries are at high risk for delayed, progressive neurological and psychiatric deterioration [1-9]. This syndrome is termed chronic traumatic encephalopathy (CTE) [1, 7, 10], and is also known as dementia pugilistica [3, 11] or 'punch drunk' syndrome [9, 12]. US military personnel [13, 14] and others who have sustained multiple concussive traumatic brain injuries [15-17] may also be at risk for this condition. Currently, there are no methods to identify progressive tau pathology in living humans. **Hypothesis:** Aggregated forms of hyperphosphorylated tau protein formed acutely in the setting of traumatic brain injury can seed further aggregation of intracellular tau in nearby cells, leading to delayed propagation of tau pathology and neurodegeneration. **Objective:** To develop standardized, high-throughput blood and cerebrospinal fluid assays for aggregated forms of tau responsible for propagation of tau pathology after traumatic brain injury.
Progress to date: The major year 1 goal for the Brody lab was to determine which mouse model of experimental TBI and which human tau transgenic mouse line would be most useful for these experiments. We have determined that controlled cortical impact in 3xTg-AD mice will be optimal. The major year 1 goal for the Diamond lab was to refine and standardize the tau propagation assay. We have increased the sensitivity of the assay by nearly 1000 fold using a flow-cytometry based assay and established quantitative standard curves.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**		12	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION:

Athletes in contact sports who have sustained multiple concussive traumatic brain injuries are at high risk for delayed, progressive neurological and psychiatric deterioration [1-9]. This syndrome is termed chronic traumatic encephalopathy (CTE) [1, 7, 10], and is also known as dementia pugilistica [3, 11] or 'punch drunk' syndrome [9, 12]. US military personnel [13, 14] and others who have sustained multiple concussive traumatic brain injuries [15-17] may also be at risk for this condition. Hyperphosphorylation and aggregation of tau protein are key pathological features of chronic traumatic encephalopathy, but at present they can only be observed post-mortem [1, 3, 6, 18-20]. Tau pathology has also been observed after single more severe traumatic brain injuries [21-23]. Currently, there are no methods to identify progressive tau pathology in living humans. The progressive aspect of chronic traumatic encephalopathy suggests that repetitive injuries may trigger an ongoing degenerative process similar to other diseases characterized by progressive tau pathology such as Alzheimer disease and frontotemporal dementia. A leading hypothesis regarding the progression of tau pathology in Alzheimer disease and frontotemporal dementia is that tau aggregates formed in one cell can propagate by exiting that cell and entering anatomically connected cells to induce tau aggregation in these cells [24-30]. While the tau pathology in chronic traumatic encephalopathy is distinct from other diseases, the propagation model offers a new conceptual framework to test these ideas in chronic traumatic encephalopathy.

The development of diagnostic tests and approaches to monitor the progression of post-traumatic tau pathology has been limited both by the lack of an appropriate small animal model and the lack of a suitable assay system. The Brody lab has recently developed a small animal model. Specifically, controlled cortical impact traumatic brain injury in 3xTg-AD and TauP301L mice, two transgenic mouse lines overexpressing mutant forms of human tau, caused tau aggregation and hyperphosphorylation in the fimbria, amygdala and hippocampi [31-33]. In parallel, the Diamond lab has recently developed cultured cell-based assays that can be used to monitor tau aggregation and trans-cellular propagation of aggregation. These assays can detect the effects of extracellular tau aggregates that cause fibril formation of native intracellular tau in cultured cells (i.e. 'seeding'), and can also track the movement of aggregates between cells [25, 26]. These advances now create an opportunity to test key hypotheses about the pathogenesis of chronic traumatic encephalopathy for the first time. In turn, a detailed understanding of this pathogenesis in experimental settings may lead to the rational development of conceptually novel diagnostic tests and therapeutic approaches in humans.

Hypothesis: Aggregated forms of hyperphosphorylated tau protein formed acutely in the setting of traumatic brain injury can seed further aggregation of intracellular tau in nearby cells, leading to delayed propagation of tau pathology and neurodegeneration.

Objective: To develop standardized, high-throughput blood and cerebrospinal fluid assays for aggregated forms of tau responsible for propagation of tau pathology after traumatic brain injury.

Specific Aims

TASK 1: *To assess extracts from the brains of tau transgenic mice subjected to experimental traumatic brain injury for tau aggregating activity using a cultured-cell based assay.*

TASK 2: *To determine whether mouse blood and cerebrospinal fluid tau aggregating activity quantitatively predict brain tau pathology and neurodegeneration in mice subjected to experimental traumatic brain injury*

TASK 3*: To test whether antibodies that block tau aggregating activity in cultured cell-based assays also block tau pathology, neurodegeneration and behavioral deficits in mice subjected to experimental traumatic brain injury*

TASK 4: *To develop an antibody-based assay for tau aggregating activity*

BODY:

We have made progress on Tasks 1 and 4.

To summarize progress on Task 1, we have found that repetitive concussive TBI does not appear to reliably accelerate tau pathology in young transgenic mice, whereas controlled cortical impact TBI causes a transient but not progressive increase in tau pathology.

Specifically, we have performed the following experiments and found the following results:

1) Controlled cortical impact TBI in 6 month old 3xTg-AD mice causes an increase in tau immunoreactivity at 1 day and 7 days after injury, but 6 months after injury this tau pathology is largely resolved. At 1 day and 7 days, the pathology is apparent in ipsilateral fimbria, ipsilateral amygdala, and contralateral hippocampus.
2) Repetitive concussive (2x- 24 hours apart) TBI in 6-8 week old hTau mice does not cause any increase in tau immunoreactivity detectible at 7 days or 6 weeks after injury. None of the mice at these ages have any abnormal tau immunoreactivity.
3) Repetitive concussive (4x injury, 24 hours apart) TBI in 6-8 week old hTau mice did not cause any increase in tau immunoreactivity detectible 6 months after injury. Both groups had quantitatively similar CP13 anti-tau immunoreactivity in subgranular layers of dentate gyrus, as assessed using blinded unbiased stereology (Figure 1). Figure 1

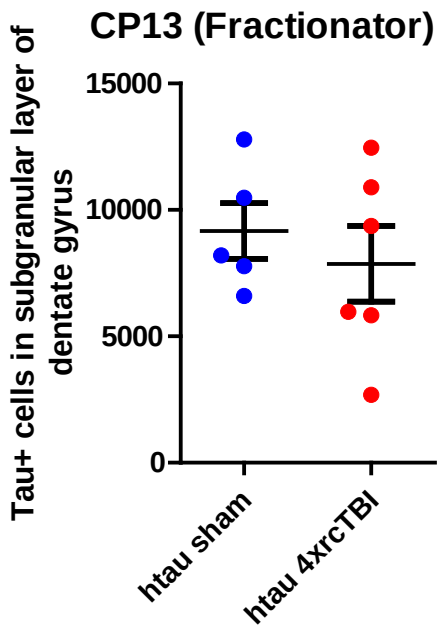


Figure 1

This 4x injury paradigm caused substantial axonal injury, as evidenced by silver staining in the ipsilateral corpus callosum and external capsule (Figure 2). However, the extent of silver staining did not differ as a function of genotype: hTau mice expressing full length human tau had no difference from tau knockout mice in blinded quantitative densitometry assessments.

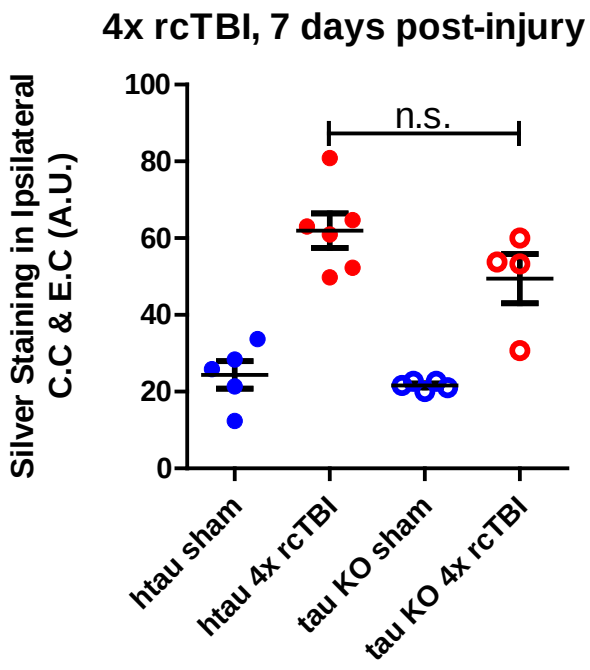
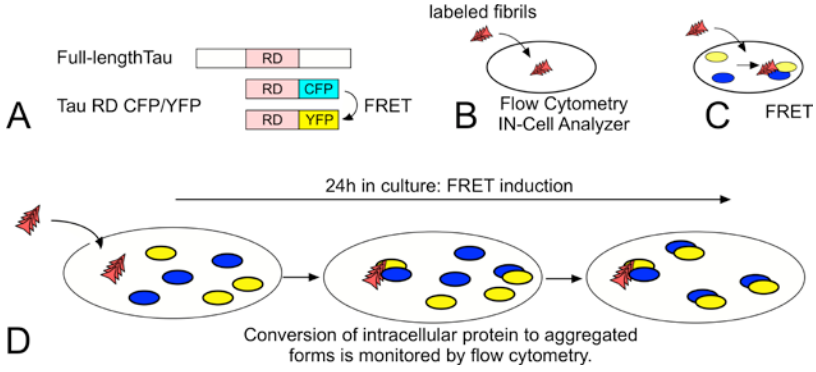


Figure 2

4) Repetitive concussive (4x injury, 24 hours apart) TBI in 6-8 week old tau P301S mice does not cause any increase in tau immunoreactivity detectible at 7 days or 6 weeks after injury. Litter-to-litter variability in the extent of tau immunoreactivity make quantitative assessments in this line of mice challenging.

As part of the development of Aim 1, we have put new effort into refining our ability to detect tau seeding activity. This is because we realized that the plate reader-based assay we were using had important limitations, both in sensitivity, and in its use to characterize many samples sensitively and precisely. The new assay was based on development of the original, but with important improvements. The assay uses a fragment of the tau protein consisting of the "repeat domain" (RD) containing a single, disease-associated mutation (P301S). This mutation predisposes the tau protein to readily aggregate. When fused to cyan and yellow fluorescent proteins (tau-CFP or tau-YFP) this tau protein serves as a "biosensor" that, when expressed in cells, enables a response to tau seeds on the cell exterior. These extracellular seeds are transported into the cell via macropinocytosis, where they trigger aggregation of tau-CFP/YFP. This aggregation event is likely to be catastrophic within the cell, meaning that once a cell converts, virtually all of the tau forms aggregates (Figure 3).

Figure 3: Tau constructs and methods of analysis. (**A**) Schematic of constructs used. Full-length tau and the repeat domain (RD) region. RD fusions to CFP and YFP allow quantification of intracellular aggregation using FRET between CFP and YFP. (**B**) Recombinant RD and full-length tau fibrils are labeled with AlexaFluor to quantify cell uptake and binding using flow cytometry or high content microscopy. (**C**) Recombinant fibrils added to cells expressing tau RD-CFP/YFP induce aggregation that is measured by FRET. (**D**) Flowchart of biosensor system to detect proteopathic "seeds."



Previously, we monitored this process using a fluorescence plate reader. In this case, the aggregation events were read out as an average across the entire well in 96-well plate. We created two innovations to improve this system. First, we created stable cell lines expressing tau-CFP/YFP. We selected our biosensor cell line to eliminate any background aggregation. Second, instead of using a plate reader, we adapted a flow cytometer to monitor protein aggregation within single cells. This dramatically increased the sensitivity and dynamic range or our assay, since we were able

to detect single cell conversion events within a population of ~120,000 cells. The increase in sensitivity vs. the plate reader system is approximately 10,000x, and the dynamic range increase is approximately 100x (Figure 4).

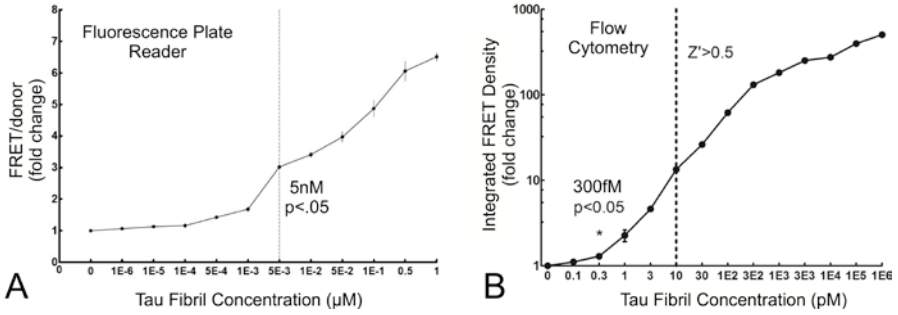


Figure 4: Detection of specific tau seeding activity. (**A**) Stable HEK/tau-CFP/YFP reporter cells were grown in a 96-well plate and exposed to the indicated range of tau fibrils. 48h later cells were fixed and FRET levels determined by FPR. Significant induction of FRET signal occurred at 5nM fibrils. (**B**) Stable HEK/tau-CFP/YFP reporter cells were exposed to a range of recombinant RD tau fibrils, followed by flow cytometry to monitor induced FRET. There was a ~1000-fold increase in the number of FRET-positive cells over the range of concentrations. The lowest concentration reliably detected ($p<.05$) was 300fM tau. At 10pM, the Z' value of the assay was >0.5. Integrated FRET Density refers to the average FRET signal from each individual cell x the number of positive cells, relative to baseline signal. Error bars=SEM. As part of the analysis of this assay, we have used it to analyze the brains of P301S transgenic tauopathy mice, which develop gradually progressive pathology over many months. These mice have been extensively characterized in our group for other purposes (REF: Yanamandra et al. Neuron 2013). We know that they develop evidence of neurofibrillary tau pathology between the ages of 4-5mos. This is associated with biochemical evidence of tau accumulation around 9mos, when detergent-insoluble protein becomes detectable. We used the seeding assay to characterize these animals, testing whether the biosensor system could robustly anticipate the development of *bona fide* neuropathology. We sacrificed P301S mice at various ages, and analyzed brain homogenates for the presence of tau pathology. With the new seeding assay, we observed clear evidence of tau pathology at ~6weeks of ages, well before the development of classical neuropathology (Figure 5).

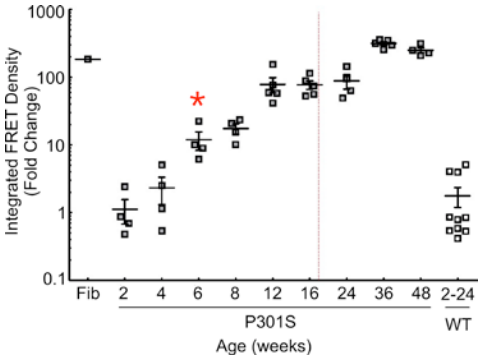


Figure 5: Seeding activity from cortex over time. P301S mice were killed at different ages as indicated, and the seeding activity was quantified in the cortex, using the HEK/tau-CFP/YFP biosensor cell line. Consistent seeding activity was detected at age 6 weeks ($p<.0001$) in P301S mice. Brains from WT mice age 2-24 weeks had no significant activity (right-most points). The dotted vertical line indicates the approximate point at which tau tangles are detected by standard histopathology. Fibrils (Fib) are used as a positive control (left-most points). Note the log scale on the Y axis, indicating the enormous range of seeding activity present over time in P301S mice.

The Diamond lab has refined the tau aggregation assays substantially since the time of the initial application. This has resulted in reliable and quantitative standard curves using recombinant 4 repeat domain (4RD) tau fibrils. In the refined flow-cytometry-based assays, tau aggregation induced in transfected HEK293 cells is expressed as integrated FRET (Fluorescence Resonance Energy Transfer) intensity.

However, even using the refined, quantitatively precise assays, we have not observed any reproducible induction of tau aggregating activity by experimental TBI. Specifically, we have found the following:

1) Controlled cortical impact TBI in 6 month old 3xTg-AD mice did not cause any increase in tau aggregation at either 1 day or 7 days after injury compared to sham 3xTg-AD mice.
2) Repetitive concussive (2x injury, 24 hours apart) TBI in 6-8 week old hTau mice did not reliably increase tau aggregation activity in hippocampal or cortical lysates. When we repeated assessments of the hippocampal lysates presented in Table 1 of progress report #1 using the more sensitive assay, we found no significant increase in tau aggregation activity in the injured mice.
3) Repetitive concussive (4x injury, 24 hours apart) TBI in 6-8 week old tau P301S mice does not cause any increase in tau aggregation in either hippocampal lysates, cortical lysates, blood or cerebrospinal fluid 7 days after injury. In one experiment, there was a trend towards reduced tau aggregation in the injured mice compared with the

sham mice in hippocampal and cortical lysates (Figure 6).

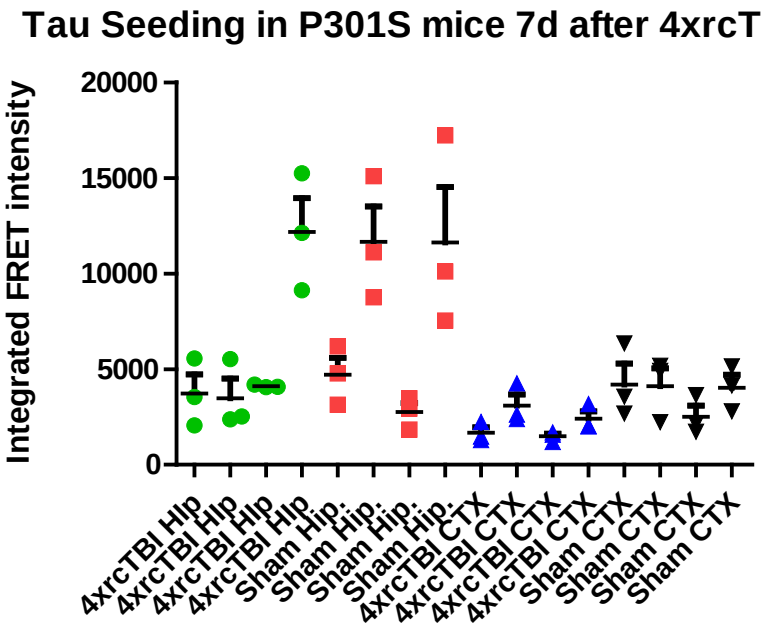


Figure 6

4) Repetitive concussive (4x injury, 24 hours apart) TBI in 6-8 week old tau P301S mice does not cause any increase in tau aggregation in either hippocampal lysates, cortical lysates, blood or cerebrospinal fluid 3 months after injury (Figure 7)

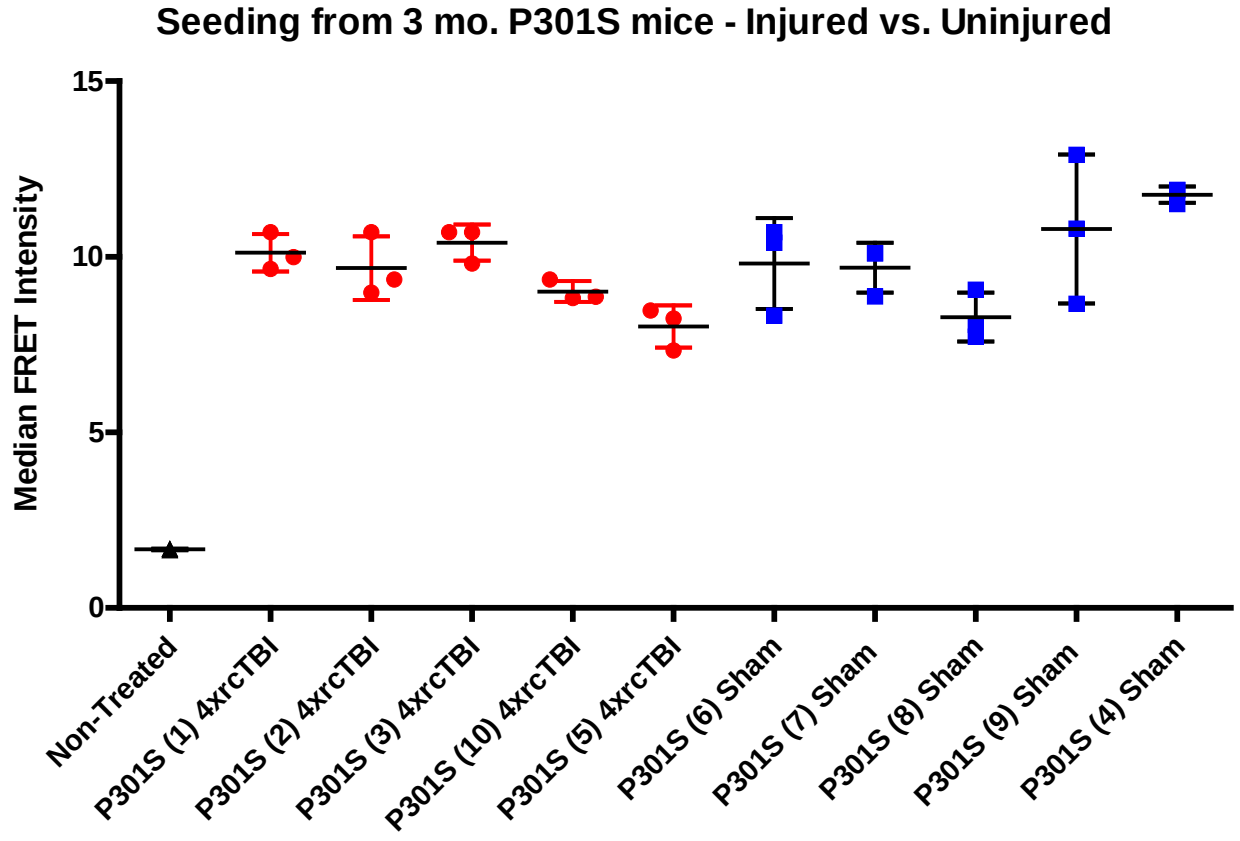
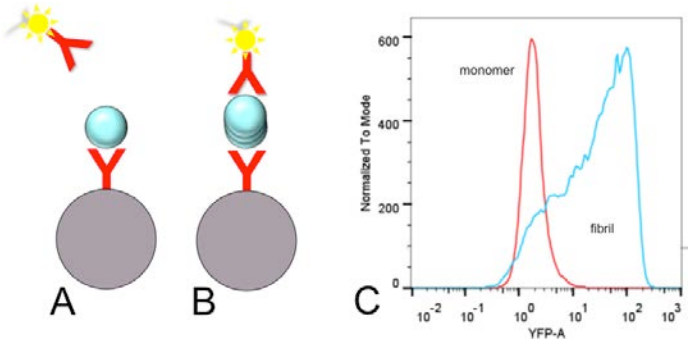


Figure 7

Progress on Aim 4:

We have been developing a bead-based detection method to monitor the development of tau aggregation. This exploits the flow cytometer to monitor the production of aggregates trapped on nano-particles that flow past a detector. In brief, a nanoparticle of approximately 100nm diameter is coated with a single anti-tau monoclonal antibody. This bead is then exposed to material containing tau aggregates. A detection antibody containing the identical antibody labeled with a fluorescent dye (e.g. Alexa488) is incubated with the sample. The bead:Ag:Ab complex is detected by flow cytometry. Preliminary evidence indicates that this "sandwich" detection method provides robust discrimination between populations of recombinant monomer vs. fibrils (Figure 8). We have not yet determined the sensitivity of this assay, nor have we used it yet in mouse models of tauopathy.

Figure 8: In vitro detection of tau aggregates. Nanoparticles are coated with anti-tau monoclonal antibody (HJ9.3) that was exposed to tau monomer (A) or tau fibrils (B). Particles were then exposed to the same antibody linked to a fluorescent dye (Alexa488), washed and fluorescence intensity per bead was assessed by flow cytometry. (C) Cytometry shows a clear discrimination of beads exposed to monomer vs. fibrils. This indicates that it may be possible to use bead-based flow cytometry systems to monitor tau aggregate production.



Summary: Thus, the major finding from the first year of the project is that in all mice tested to date, tau pathology induced by TBI is a transient phenomenon that does not appear to propagate or increase over time. This raises a major question: What are the mechanisms underlying clearance of tau pathology in these young mice? It is possible that any tau pathology arising in the repetitive concussive models could have been rapidly cleared and therefore not detected by our immunohistochemical methods.

Our plan for the next project period is to investigate the mechanisms underlying tau clearance using the most promising mouse model: 3xTg-AD mice injured using controlled cortical impact. Hypotheses to be tested include that dephosphorylation by protein phosphetases leads to disaggregation of tau, ubiquitination leads to degredation of tau, and inductions of autophagy leads to lysosomal degradation of tau aggregates.[34] It may be that tau is cleared more aggressively in the brains of mice than in the brains of humans. If so, inhibition of one or more of the endogenous tau clearance mechanisms will be required to recapitulate progressive tau pathology in a mouse model following TBI to accurately recapitulate the human phenotype.

In all animals, clearance of protein aggregates declines with age. Therefore, in parallel, we will test the hypothesis that repetitive concussive injuries in older mice (6-12 months) may cause progressive tau pathology. Towards this end, we have begun characterizing a 5x repetitive closed skull injury model using a midline 1 mm metal tip impact as developed by the Crawford lab[35] . Our current repetitive concussive injury model involves a 3.3 mm rubber tip impact which does not cause concussive TBI in mice older than 8 weeks of age (TJ Esparza, RE Bennett, DL Brody, unpublished data) due presumably to their thicker skulls.

A second major finding from the first year of the project is that tau aggregation activity measured by FRET may not necessarily always reflect TBI-related tau pathology. Accordingly, we plan to extend our search for a blood or CSF-based marker for tau pathology using mass-spectrometry based discovery of tau post-translational modifications. If successful, one or more characteristic tau post-translational modifications could be targeted for an ELISA-type assay which would be appropriate for widespread use.

KEY RESEARCH ACCOMPLISHMENTS:

1) Controlled cortical impact injury in 6 month old 3xTg-AD mice cause a transient but not progressive increase in tau pathology. However, repetitive concussive injury in 6-8 week old hTau and P301S did not.
2) Sensitivity of tau aggregation assays has improved substantially. However, the tau aggregation assays have not correlated fully with tauopathy.

REPORTABLE OUTCOMES:

None

CONCLUSION:

It appears that it will be more challenging than expected to recapitulate human TBI-related tau pathology using transgenic mouse models. Nonetheless, significant technical advances have been made and there are several feasible paths forward which if successful, will allow development a pharmacodynamically useful preclinical model and non-invasive blood or CSF biomarker for TBI-related tauopathy.

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Appendices: None.